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Epithelial phosphate transport in ruminants, mechanisms and regulation

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The uptake of phosphate (Pi) into cells and its assimilation into cellular material are essential features of metabolism. In ruminants, secretion of Pi via the parotid saliva and the subsequent reabsorption in the small intestine are essential aspects of the massive recycling of Pi. Pi serves both as a buffer against the volatile fatty acids produced in the rumen by microbial fermentation, and as a nutrient for the rumen microorganisms.

Dietary Pi deficiency in ruminants has been shown to lead to reduced rumen microbial protein synthesis, reduced growth rate, disturbances in reproduction and lactation, and reduced bone mineralization. Pi deficiency is the predominant mineral imbalance affecting ruminants in many parts of the world [1–3].

Ruminants consuming roughage diets such as hay, straw and grass usually excrete little Pi in the urine and are more dependent on the gut rather than the kidney for major adjustments in Pi-homeostasis. Fecal Pi is the main excretory pathway for Pi; this is derived mainly from saliva [4, 5]. This situation is in contrast to that seen in non-ruminants, as well as pre-ruminants, where the kidney is the major control site for maintenance of plasma phosphate.

In ruminants fed concentrate diets, however, significant Pi is excreted by the kidneys. The reason for this is unclear, although it has been suggested that this is due to alterations in salivary flow rate. A reduction in the salivary flow rate in ruminants fed concentrate diets results in a high plasma Pi and excess Pi is excreted via the kidneys. The physical nature of the diet is shown to be the major factor influencing the salivary flow rate and hence producing a renal response [5].

This paper describes the mechanisms and properties of Pi transport systems in ovine intestine, parotid gland and kidney. The approaches towards structural identity of these transporters will be discussed. The accumulated information is to serve as a baseline for further studies to identify the structure of these transporters. Such studies will facilitate a better understanding of the molecular mechanisms which underlie the control of Pi homeostasis in ruminants.

Phosphate recycling in ruminants

In ruminants the salivary glands are the major organs responsible for endogenous Pi secretion entering the gut. The daily secretion rate is 10 to 16 liters per day for sheep and 30 to 50 liters in cows, containing 16 to 40 mmol/liter orthophosphate, that is, about 200 to 300 mmol/day [6–8]. This high concentration of Pi is achieved both by the high salivary flow rate, and the ability of the parotid acinar cells to concentrate Pi in comparison to plasma Pi.

This amount of Pi greatly exceeds that supplied by the diet (50 to 60 mmol/day), and therefore it is essential that there is an efficient scavenging system for the absorption of Pi from the gut. Hence the mechanism of absorption of Pi in the gut and secretion via saliva in ruminants are of major significance.

The majority of published studies point to the proximal small intestine, where the pH of the digesta is acid, as the major site of Pi absorption in both sheep [4, 9] and cattle [10, 11]. In non-ruminants, the acid chyme which leaves the stomach is neutralized by the bile, pyloric, duodenal and pancreatic secretions. In contrast, the proximal small intestinal contents of sheep and cattle are usually markedly acid, having a pH of a 3 to 4 [12, 13]. It has been shown that the neutralizing capacities of the intestinal secretions are insufficient to deal with the relatively large volume of digesta leaving the abomasum [12].

Comparative aspects of intestinal Pi transport in ruminants and non-ruminants

The transport of Pi in the intestinal brush-border membrane of most non-ruminant species studied, is via a Na/Pi cotransporter [14, 15], with the highest rate of absorption occurring in the duodenum [14, 16]. The Na/Pi cotransporter has been well characterized by transport experiments using brush-border membrane vesicles isolated from the small intestine of a variety of species. The results have indicated that the intestinal brush-border Na/Pi cotransporter exhibits an apparent K_m for Pi of the order of 0.05 to 0.2 mM and a stoichiometry (Na^+/Pi) of 2:1, with higher uptake rates at pH 6 compared to pH 7.5 [14, 17, 18].

The ovine and bovine duodenal brush-border membrane vesicles have also been used to investigate the mechanism of Pi transport. As can be seen in Figure 1, the imposition of a pH gradient, $pH_{out} < pH_{in}$, would support the uptake of Pi into membrane vesicles. The transport of Pi into membrane vesicles is concentrative, showing a typical overshoot, implying that the movement of Pi is energized by an inwardly directed H^+ gradient [19, 20].

The H^+ energized uptake is independent of extravesicular Na^+, and inclusion of other cations such as K^+, Li^+ and choline$^+$ in the

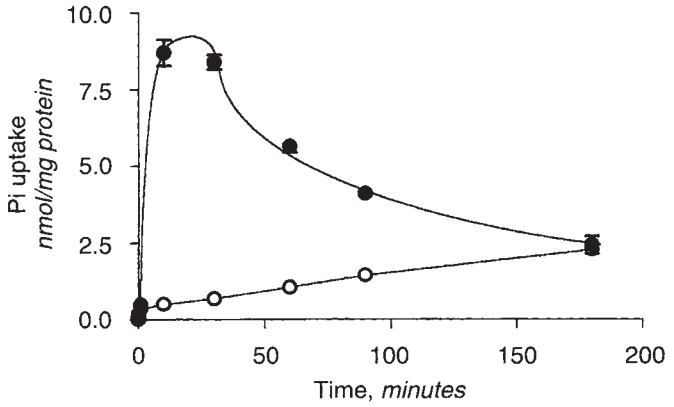

Fig. 1. *Time course of H^+-dependent transport of Pi into ovine duodenal brush-border membrane vesicles.* Imposition of pH gradient across the vesicular membrane, pH_{out} = 5.5 and pH_{in} = 7.5, energizes the uptake of Pi into membrane vesicles (■). No significant uptake is observed (○), when there is no pH gradient across the vesicular membrane, pH_{out} = pH_{in} = 7.5.

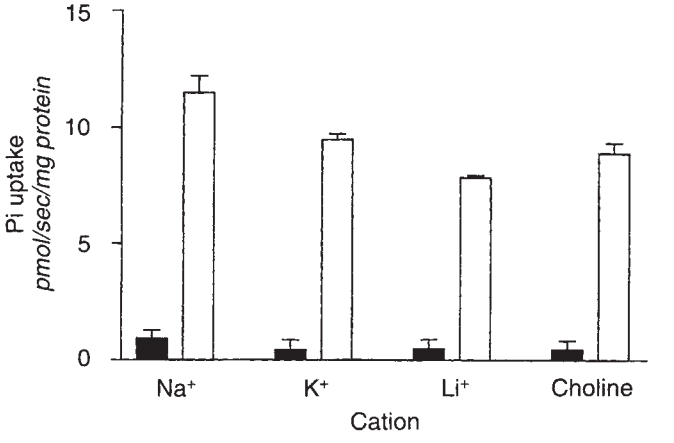

Fig. 2. *Effect of cations on the H^+-dependent transport of Pi by ovine duodenal brush-border membrane vesicles.* No significant uptake of Pi into membrane vesicles is observed when there in no pH gradient across the vesicular membrane (■). Imposition of a pH gradient across the vesicular membrane energizes the uptake of Pi (□). This uptake is independent of the monovalent cations such as Na^+, K^+, Li^+ and choline$^+$ present in the incubation media.

media support the uptake to the same level as seen in the presence of Na^+ (Fig. 2).

Using compounds that are capable of dissipating the imposed H^+-gradient inhibits the uptake of phosphate into ovine duodenal brush-border membrane vesicles see Figure 3. For example, inclusion of 4,5,6,7-tetrachloro-2-trifluoromethylbenzimidazole (TTFB), a classical mitochondrial uncoupling agent [21], in the incubation media inhibited H^+-dependent Pi uptake (Fig. 3). Furthermore, nigericin, a carboxylic ionophore that preferentially catalyzes the electroneutral exchange of K^+ for H^+ [22], inhibited H^+-dependent Pi uptake into K^+ loaded membrane vesicles (Fig. 3). The accumulated data support the proposal that the uptake of Pi into the ovine/bovine duodenal brush-border membrane vesicles is by a H^+/Pi cotransport mechanism.

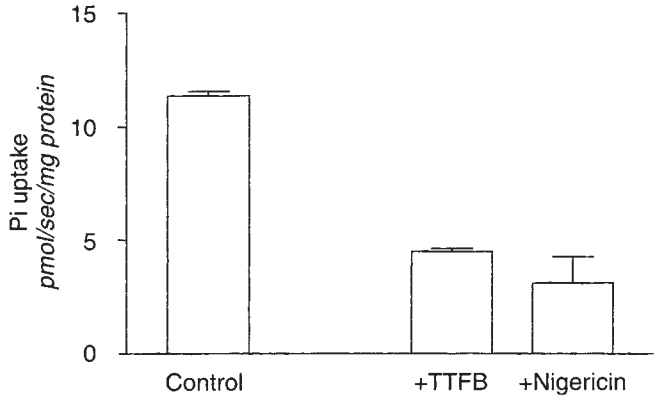

Fig. 3. *Inhibition of H^+-dependent transport of Pi in ovine duodenal brush-border membrane vesicles.* Transport of Pi was inhibited by abolishing the pH gradient with either TTFB (6.5 μg/mg protein), or nigericin (7.2 μg/mg protein).

The kinetic parameters of H^+/Pi cotransport in ovine duodenal brush-border membrane vesicles exhibit an apparent K_m for Pi of 0.65 ± 0.06 mM and V_{max} of 364 ± 16.6 pmol/sec/mg protein, at pH 5.5. The studies directed to modify the membrane potential across the vesicular membrane, either by inclusion of valinomycin and K^+, or anions of different permeability, indicated that the transport of Pi across the intestinal brush-border membrane in sheep is not associated with the net transfer of electrical charge [23]. The pH at the site of Pi transport in ovine intestine is 3 to 4 [12]. At this pH the majority (98%) of the orthophosphate is in the monovalent form, suggesting that the species to be transported is $H_2PO_4^-$. It has been shown that in the intestine of non-ruminant species, the monovalent anion is also the preferred transported species [24–26].

The existence of the H^+ rather than Na^+-coupling for Pi transport in the ruminant intestine may represent the use of an alternative cation substrate. A similar situation has been shown for the intestinal Na^+/glucose cotransporter (SGLT1), which normally has an overwhelming preference for Na^+ to energize the movements of glucose. Using electrophysiological measurements with a cloned SGLT1 expressed in *Xenopus* oocytes, Hirayama, Loo and Wright [27] have shown that H^+ can substitute for Na^+ in driving sugar transport through SGLT1. Some strains of bacteria can also utilize either Na^+ or H^+ to energize the movement of solutes, depending on the composition and the pH of the growth media [28]. Therefore, it could be that the difference in the cation specificity of the Pi transporter in the intestine of ruminant and non-ruminant species is a mechanistic adaptation and a structural modification in the cation binding sites of Pi transporters in response to the lumenal pH.

The information on the amino acid sequence and the substrate recognition site of intestinal Pi transporters in ruminants and non-ruminants would permit clarification of this difference. Unfortunately, as yet no information on the molecular structure of intestinal phosphate transporters is available.

Effects of dietary Pi restriction on ovine intestinal Pi transport

Dietary Pi restriction in ruminants is associated with a significant increase in the capacity of the small intestine to absorb Pi, as evidenced by three- to fourfold increase in the maximum velocity

Table 1. Kinetic parameters of Pi uptake by brush-border membrane vesicles isolated from the intestine of sheep fed low-Pi or high-Pi diets

Diet	Km (mM)	V_{max} *pmol/sec/mg protein*
Low Pi	0.59 ± 0.12	708 ± 119
High Pi	0.65 ± 0.06	364 ± 17
P	NS	< 0.001

of pH stimulated Pi uptake [20]. However, the affinity of the transporter for the substrate is not changed, suggesting that the mechanism of uptake is not affected (Table 1). This enhancement in the transport activity is independent of external factors such as circulating levels of parathyroid hormone, Ca^{2+}, growth factors and 1,25 hydroxycholecalciferol [1,25$(OH)_2$D3] [20, 29–31], and seems to represent an intrinsic adaptive response of the small intestine to a low Pi diet. Therefore, the ruminant intestine provides an ideal system for studies to define the basic molecular mechanisms that are involved in the adaptation of the intestinal tract to variations in the Pi content of the diet.

Dietary Pi deprivation also enhances small intestinal Pi absorption in non-ruminants. However, the increased intestinal absorption of Pi caused by a low Pi diet correlates in time with the rise in 1,25$(OH)_2$D3 level, suggesting that the hormonal form of vitamin D is implicated in the intestinal adaptive response in non-ruminants [16].

Towards structural identification of intestinal Pi transporter

Using *Xenopus laevis* oocytes as an expression system, three Na^+/Pi cotransporters have been cloned from cDNA libraries of rabbit (NaPi-1), rat (NaPi-2) and human (NaPi-3) kidney cortex [32, 33].

Comparison of amino acid sequences revealed an 80% identity between NaPi-2 and NaPi-3 but only 20% identity between NaPi-1 and NaPi-2/3. It has been proposed that the cloned Na^+/Pi cotransporters belong to different types of epithelial Na^+-dependent transporters for inorganic phosphate; Type I represented by NaPi-1 rabbit and a Type II, represented by NaPi-2 rat and NaPi-3 human. Both types appear to be exclusively expressed in kidney cortex [34]. There is no cross-hybridization of these cDNA probes to non-ruminant small intestinal mRNA [15]. Equally no cross-hybridization of these cDNA probes to mRNA species isolated from the ovine intestine has been detected (Wood and Shirazi-Beechey, unpublished observation). It is therefore concluded that the intestinal brush-border phosphate transporters may belong to a different class of Pi transporters.

In an initial step towards the isolation of the cDNA for the ovine intestinal Pi transporter, the mRNA isolated from the ovine proximal intestine has been expressed in *Xenopus* oocytes. As can be seen in Figure 4, the injection of ovine duodenal mRNA into oocytes resulted in a sixfold increase in the rate of the H^+-dependent uptake of ^{32}P-orthophosphate compared to control oocytes.

This uptake was inhibited by 79% in the presence of 2 mM phosphonoformate (pfa), a competitive inhibitor of this transport protein. Abolishing the pH gradient across the oocyte plasma membrane (that is, changing the pH of assay medium from 5.5 to 7.5) resulted in a rate of uptake similar to that seen in control oocytes. The low levels of Pi uptake observed in water injected oocytes (control) were the same in the presence or absence of pH

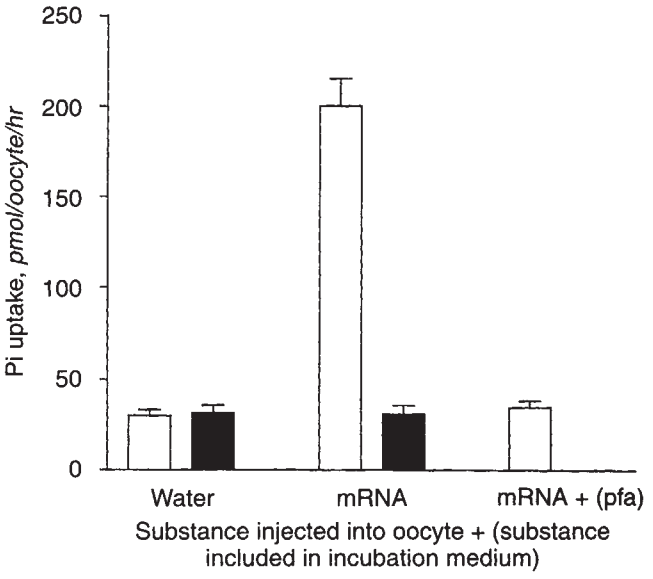

Fig. 4. *Functional expression of H^+-dependent Pi transport in Xenopus laevis oocytes.* Individual oocytes were injected with ovine duodenal mRNA and then incubated for five days at room temperature, prior to measurement of Pi transport. Pi transport was measured in incubation media of either pH 7.5 (■) or pH 5.5 (□). Inhibition of Pi transport was achieved by abolishing the pH gradient (■) or by including 2 mM pfa in the incubation medium.

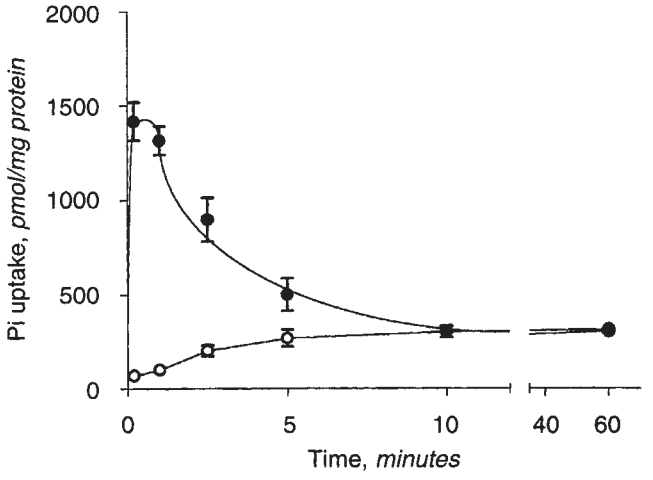

Fig. 5. *Time course of Na^+-dependent Pi transport by ovine parotid basolateral membrane vesicles.* Inclusion of Na^+ in the incubation medium, 100 mM out, 0 mM in, energizes the uptake of Pi into membrane vesicles (●). No significant uptake is observed (○), when Na^+ is replaced by K^+.

gradient across the oocyte membrane. The latter results indicate that there is no detectable endogenous H^+-dependent Pi transport in the plasma membrane of the oocyte. These results provide a basis for the expression cloning of the ovine intestinal H^+/Pi co-transporter.

Phosphate transport in the ovine parotid gland

Ruminants secrete large volumes of a well buffered and isotonic saliva. The parotid glands account for 50% of that produced [6].

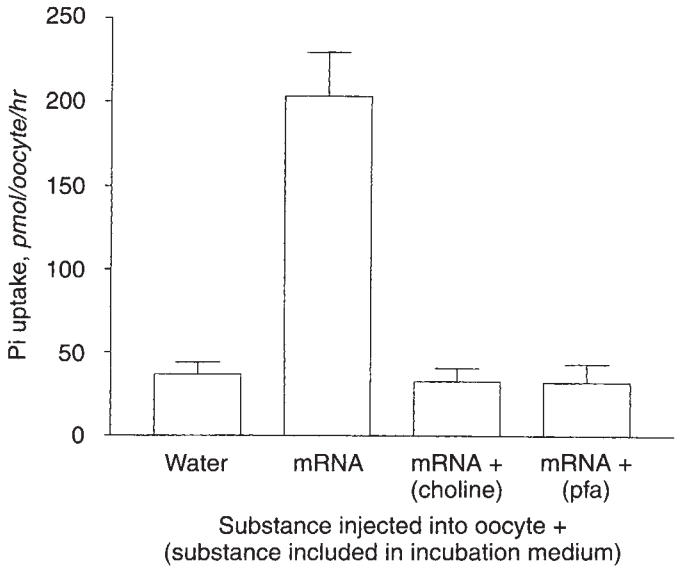

Fig. 6. *Functional expression of Na^+-dependent Pi transport in Xenopus laevis oocytes.* Individual oocytes were injected with ovine parotid mRNA and then incubated for five days at room temperature, prior to measurement of Pi transport. Inhibition of Pi transport was achieved by replacing Na^+ with choline or by including 5 mM phosphonoformate (pfa) in the incubation medium.

The concentration of Pi in the parotid secretion, 16 to 40 mM, is remarkably high. This salivary Pi is the result of secretion by the parotid acinar cells [35]. The similarities of the Pi concentrations in the final and primary saliva makes it unlikely that there is ductal secretion of this anion, and that virtually all Pi appearing in the final saliva is derived from the primary secretion [35].

The flux of Pi from the blood stream into the parotid acinar cell and the subsequent extrusion from the cell to the primary saliva is, overall, an energy requiring process. The movement of the negatively charged Pi ions through the cell also imposes a requirement for compensation of the electrical charge. The mechanism by which Pi is transported from blood into the acinar cells of the parotid gland has been investigated. Using purified and well-chracterized basolateral membrane vesicles isolated from the ovine parotid acinar cells [36, 37], it has been shown that the movement of Pi into the vesicles is energized by a Na^+-gradient (Fig. 5). The observed uptake was reduced by 70 to 80% in the presence of 5 mM phosphonoformate, a competitive inhibitor of Pi transport. When the Na^+ gradient was replaced with a K^+ gradient, no uptake was observed (Fig. 5) [36]. It was concluded that the Pi uptake was driven by a Na^+-dependent Pi transporter located on the basolateral membrane of the acinar cell [36].

Nothing is known about factors which regulate the activity and the expression of the parotid gland Na/Pi cotransporter.

The microinjection of the *Xenopus* oocytes with the ovine parotid gland mRNA has led into the functional expression of the Na^+-dependent Pi transporter in the plasmalemma membrane of the oocyte (Fig. 6) [38]. Injection of ovine parotid gland mRNA resulted in a rate of Pi uptake sixfold greater than that observed in control oocytes injected with water. The observed Pi uptake in water injected oocytes has been attributed to intrinsic Na^+-dependent Pi transport activity present in the plasma membrane of oocytes [32]. In the presence of 5 mM phosphonoformate the rates of uptake were reduced by 72%. When the Na^+-gradient was replaced with choline, the rates of uptake were equal to that of the control (Fig. 6). The expressed transporter displays similar properties in respect to Na^+-dependency and inhibitor sensitivity to that observed in native ovine basolateral membrane vesicles [36].

The functional expression of the parotid acinar cell Na^+-dependent Pi transporter in oocytes, indicated the presence of transcript(s) encoding this protein within the isolated RNA. The potential of cDNA probes encoding type I and type II renal Pi transporters to hybridize to the ovine parotid gland mRNA were investigated. The results of these studies showed that the parotid Na^+-dependent Pi transporter could belong to the type II class of Pi transporter proteins.

Northern blot analysis of ovine parotid RNA, using renal Na/Pi transporter cDNA probes type I and type II, showed that there was no cross reactivity between parotid gland mRNA and the cDNA encoding NaPi-1. However, the cDNA encoding the NaPi-3 hybridized to a major transcript at 4 Kb. Cross reactions were also seen at 2.3, 1.3 and 1 kb. The NaPi-3 cDNA could provide a molecular probe to screen an ovine parotid gland cDNA library for clones with sequence homology to NaPi-3.

No studies have been carried out on the mechanism by which Pi is transported from the ovine parotid acinar cell into the primary saliva. The lumenal membrane of the pyramidal-shaped parotid gland acinar cells are an order of magnitude smaller to that of basolateral membrane [39]. This causes technical problems in isolating lumenal membrane vesicles for the identification of the mechanism of Pi transport at the lumenal pole. Technologies such as patch clamping may prove to be more successful in such studies.

The flow of Pi through the lumenal membrane can be driven by the voltage gradient across the lumenal membrane. The −60 to −80 mV membrane potential is sufficient to energize the movement of Pi through the parotid acinar lumenal membrane, and concentrate it in the primary saliva. For this movement, however, the presence of a Pi channel on the lumenal membrane is a prerequisite.

Pi transport in the ruminant kidney

Ruminants fed roughage diets such as hay, straw or grass usually excrete very little Pi in their urine. Major adjustments in Pi homeostasis are achieved via the gut, through changes in both salivary Pi secretion and in the efficiency with which this is reabsorbed by the intestine [5]. The kidney, however, appears to be of greater importance as a route for Pi excretion in ruminants fed cereal based, or finely ground, diets. This change from gut to kidney as a route for Pi excretion is linked to a lower salivary flow rate; the secretion of Pi into the gut via the saliva is reduced. This imbalance is resolved by a higher level of Pi excreted in the urine [5].

Studies on the mechanism of Pi transport in brush-border membrane vesicles isolated from ovine kidney cortex have indicated that Pi transport is energized by a Na^+-gradient with similar kinetic characteristics to that seen in non-ruminant species, with a K_m of 0.23 ± 0.03 mM and V_{max} 16.4 ± 3.2 pmol/sec/mg protein [23]. The response to dietary Pi deprivation is manifested by a twofold increase in V_{max}, with no change in the affinity of the transporter for Pi, that is, no change in K_m [23]. The hybridization of human renal Pi transporter cDNA to the ovine cortical mRNA,

observed in Northern blot analysis, implies that the transporter may belong to the type II Pi transporters.

Structural identification of Pi transporters in the organs involved in Pi homeostasis in ruminants will enable a better understanding of the molecular mechanisms invoved in the physiological control of this vital mineral.

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